

Ketterson / Nolan Research Group Collection

This document is part of a collection that serves two purposes. First it is a public archive for data and documents resulting from evolutionary, ecological, and behavioral research conducted by the Ketterson-Nolan research group. The focus of the research is an abundant North American songbird, the dark-eyed junco, *Junco hyemalis*, and the primary sources of support have been the National Science Foundation and Indiana University. The research was conducted in collaboration with numerous colleagues and students, and the objective of this site is to preserve not only the published products of the research, but also to document the organization and people that led to the published findings. Second it is a repository for the works of Val Nolan Jr., who studied songbirds in addition to the junco: in particular the prairie warbler, *Dendroica discolor*. This site was originally compiled and organized by Eric Snajdr, Nicole Gerlach, and Ellen Ketterson.

Context Statement

This document was generated as part of a long-term biological research project on a songbird, the dark-eyed junco, conducted by the Ketterson/Nolan research group at Indiana University. For more information, please see IUScholarWorks (<https://scholarworks.iu.edu/dspace/handle/2022/7911>).

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PROTOCOL FOR STEROID RADIOIMMUNOASSAY OF EGG YOLKS

This protocol has been copied and modified from protocols provided by Dr. John Wingfield at the University of Washington and Dr. Hubert Schwabl at Washington State University. This protocol can be used to measure the concentration of steroid hormones in yolk samples. It has been written for use in the laboratory of Dr. Ellen Ketterson at Indiana University, and therefore the details may require modification if the procedure is performed elsewhere. The general procedure, which takes 5 days to complete, is described below. Variations and additional information are provided in the appendices. Prepared October 1997 by Joe Lipar.

PRIOR TO START OF ASSAY

1. Distillation of ethyl acetate.

We purchase ACS quality ethyl acetate, which requires distillation no more than 24 hours prior to the start of the assay. Pour the new or existing ethyl acetate (up to 1.5 liters) into the distillation apparatus and secure the stopper in place. Turn on the cold running water and the heating block (adjusted to 55-60°). The first and last 50 ml of distillate should be discarded. Isooctane (2,2,4-trimethylpentane) is not distilled due to its extreme flammability, so nanograde quality is purchased.

2. Double-check of materials.

Before beginning an assay, make sure that all of the following materials are available for use.

Nitrogen Gas - BOC Gas, 1-800-247-2644, Acct # N8115

Nanograde Quality 2,2,4-Trimethylpentane (Isooctane) - Fisher Scientific

Petroleum Ether - Chemistry Stockroom

Ethyl Ether - Chemistry Stockroom

Celite - NOTE as of 20 February 2004 Sigma's stock of Celite has been compromised, use Fisher Scientific's Celite Analytical Filter Aid #C211-500

Phosphate Buffer - See Appendix D

Ethylene Glycol - Sigma

Propylene Glycol - Sigma

10x75 Test Tubes - Fisher Scientific

Charcoal/Dextran Suspension - See Appendix D

Standard, Labeled Steroid, and Antibody for the particular hormone(s) being assayed - See Appendices E, F, and G

Ultima-Gold Scintillation Fluid - Packard Instrument Company, 800 Research Parkway, Meridian, CT 06450. (800-323-1891). Item # = 6013329 (\$225/2x5L Containers)

All other materials that are needed throughout the assay can be purchased at the biology stockroom. Make sure that you have everything you need if you are going to work over a weekend when the stockroom is closed.

DAY 1

1. Desiccation of celite.

Celite (diatomaceous earth) should be heated in a crucible at 1000°F (range of 950-1100°F) for 24 hours prior to use. The temperature gauge on the oven should be set at about 1.9. Be sure to pack plenty of celite into the crucible so you do not run out while packing the columns.

2. Preparation of yolk samples.

If samples have been obtained directly from viable eggs (See Appendix A then this step will have been done at that time. If samples are being taken from whole yolks, this step can be done now (or earlier if time permits).

Label an appropriate number of 1.5 ml Eppendorf tubes and pre-record their masses. Place a sample of homogenized yolk in each tube and re-record the masses. A total of 5-15 mg of yolk has been appropriate for the detection of steroids in the red-winged blackbird. Suspend the yolk sample in 500 μ l of distilled water and vortex vigorously until the sample is homogenous (the addition of 2-3 small glass beads will facilitate suspension).

3. Preparation of blank tubes.

Blank samples provide a negative control for background radiation in the assay. The first and last tubes in the assay should be blanks. Add 500 μ l of distilled water to two 1.5 ml Eppendorf tubes.

4. Preparation of standard tubes.

Standard samples consist of a known amount of the steroid being assayed. They are used to measure the accuracy of the assay and to provide a measure of interassay variation. The second and second-to-last tubes in the assay should be standards. Additional standard samples may be placed throughout the assay if desired.

The standard tubes should be prepared in 1.5 ml Eppendorf tubes. For each steroid being assayed, place 50 μ l of cold (non-radioactive) steroid in each standard tube using a Hamilton Syringe. This will be 500 pg for progesterone (P), 1000 pg for corticosterone (B), and 250 pg for testosterone (T), estradiol (E2), and dihydrotestosterone (DHT). Bring the volume up to 500 μ l with distilled water.

5. Setting up the recoveries.

A small amount of labeled steroid is now added to each sample and standard tube. Total cpm for each sample will be measured at the end of the assay to determine the percentage of labeled steroid that is recovered (i.e. not left behind during the extraction and chromatography steps), and the final concentration of steroid in each sample will be adjusted accordingly.

For each steroid being assayed add 20 μ l (2000 cpm) of labeled steroid to all samples and standard tubes (but not to the blanks). An Eppendorf repeater pipette set on 2 with a 0.5 ml tip can be used. A yellow pipette tip (designed for the Eppendorf P200 Pipetman) can be fit over the end of the 0.5 ml tip to reduce drips. Also, for each steroid add 20 μ l of hot steroid to a scintillation vial (one vial for each steroid). Add 2.5 ml Ultima-Gold scintillation fluid to this scintillation vial, vortex, cap, label, and store in a dark place. The total cpm of these vials will be measured to help determine the percentage recovered in each sample. Vortex all tubes and

refrigerate overnight to allow the hot steroids to equilibrate with the yolk lipids and binding proteins.

DAY 2

1. Extraction of steroids from yolk samples (Part 1).

-Transfer all samples into 12 ml conical glass tubes using an Eppendorf P1000 Pipetman. Rinse each sample with 500 μ l distilled water and add rinse to sample.

-Prepare the extraction solvent which consists of petroleum ether:diethyl ether in a ratio of 30:70. The diethyl ether must be freshly opened to avoid the formation of peroxides.

-Add 3 ml of extraction solvent to each sample. This can be done with an Eppendorf repeater pipette set on 4 with a 12.5 ml tip.

-Vortex each sample for approximately 10 seconds.

-Let the samples sit for 20 minutes to allow for complete phase separation. While waiting prepare a snap freezing bath of dry ice in methanol.

-Snap freeze each sample and pour off the unfrozen supernatant into a 12x75 test tube.

-Repeat the extraction and combine the extracts.

-Evaporate the samples under nitrogen gas.

-Add 1 ml 90% ethanol to each tube, vortex, and freeze covered with parafilm overnight at -20°C.

2. Preparation of chromatography columns. (For short columns see Appendix B.)

Set up an appropriate number of 5 ml pipets with rubber bands to hold them in the rack. It is a good idea to make 2-3 extra columns in case some are broken or made improperly. Place a glass bead in the bottom of each pipet. To make the water trap mix 6 grams of celite with 2 ml distilled water using a mortar and pestle. Six grams of celite will make approximately 32 water traps. (The mortar will hold up to 10 grams at a time if more is desired.) Mix until you are sure it is homogenous. Shake the celite mix into each pipet using a joint adapter/funnel. Fill to about the 3.5 ml mark, then pack firmly to the 4.5 ml mark using a glass rod. Generally, the celite mix packs to about 1/3 of the loose volume.

To make the glycol phase of the columns mix propylene glycol (1,2-propanediol) and ethylene glycol in equal amounts. Add 3 mls of glycol mix to 6 grams of celite and mix well with a mortar and pestle until homogenous. Six grams of celite will make approximately 8 long columns. Add the celite mix with the joint adapter/funnel to the 3.0 ml mark and pack down to the 4.0 ml mark. Add again to the 2.5 ml mark and pack to the 3.5 ml mark. Add once more to the 2.0 ml mark and pack to the 3.0 ml mark. The columns are now packed with 1.5 ml celite/glycol mixture on top of 0.5 ml celite/water mixture.

The columns should be wet with 4 ml isooctane so they do not dry out overnight. A 10 ml pipet may be used to do this. Attach the hoses and turn on the nitrogen gas to push the isooctane through the columns. (The flow rate may be as great as desired at this time, but the columns should never be allowed to dry out. If this does occur, the columns must be thoroughly rewet with more isooctane.) Stop when the solvent drops to within one mark of the celite. Add another 4 ml of isooctane, then attach the hoses to avoid overnight evaporation. Make sure the valves are closed and the gas is off.

DAY 3

1. Extraction of steroids from yolk samples (Part 2).

- Run the Beckman TJ-6 centrifuge for about 15 minutes to cool it to 0°C.
- Spin the samples at 2000 rpm for 5 minutes. This will pellet neutral lipids and proteins, which can interfere in the chromatography step.
- Pour the supernatant into a 13x100 test tube and dry under nitrogen gas.
- Resuspend the samples in 500 μ l 10% ethyl acetate in isooctane. The Eppendorf repeater pipette may be used.

2. Chromatography. (For short-column chromatography see Appendix B.)

Run the remaining isooctane from the previous day out of each column. Stop the flow in each column when the solvent front just reaches the top of the celite. To add the samples to the columns, first vortex a sample, then transfer it to its respective column with an Eppendorf P1000 Pipetman. Rinse the 13x100 test tube with another 0.5 ml of 10% ethyl acetate in isooctane, vortex, and add to the same column. Leave the blue pipetman tip in the top of the column to mark your place and to avoid adding two samples to the same column. When all samples have been transferred remove and discard the blue tips.

Attach the hoses to the columns and regulate the nitrogen pressure to that no single column drips faster than 1 drip every 6 seconds. Discard the eluate (by collecting in the gutter and evaporating in the hood). When the solvent reaches the top of the celite turn off that column and remove the hose. Do not allow any part of the celite to dry out.

All of the following fractions (prior to the last being collected) must be eluted, but any that are unwanted may be discarded. Fractions are collected in 13x100 test tubes placed under the columns.

To collect progesterone: Add 4 mls 2% ethyl acetate in isooctane and blow down under regulated pressure into collection tubes. If P will be discarded, use 4 ml pure isooctane.

To collect dihydrotestosterone: Add 4.5 mls 10% ethyl acetate in isooctane as above.

To collect testosterone: Add 4.5 mls 20% ethyl acetate in isooctane as above.

To collect estradiol: Add 4.5 mls 40% ethyl acetate in isooctane as above. This step is optional and may be skipped if estradiol is to be discarded.

To collect corticosterone: Add 4 mls 50% ethyl acetate in isooctane. (Use 4.5 mls if the 40% fraction was skipped.)

Evaporate the eluates under nitrogen gas in the 40°C water bath. Add 550 μ l of buffer (PBSG) to each tube for all hormones except B. To the B eluates add 1 ml buffer. The buffer can be added using a combination of tips on the Eppendorf repeater pipette. Whirl mix the racks of tubes, cover with parafilm, and refrigerate overnight. (To save a day, you may place the racks on the shaker for at least 45 minutes and proceed to set up the radioimmunoassay.)

DAY 4

This day is devoted to setting up the competitive-binding radioimmunoassay. Begin by placing the appropriate number of 10x75 test tubes in racks. First arrange 12 pairs for the

standard curve, with a space between the 3rd and 4th pairs. Follow with a pair of tubes for each sample. (When you get to the charcoal step on Day 5, only 120 tubes may be centrifuged at one time. This translates into the standard curve plus 48 samples including blanks and standards. If you have more than 48 samples, a second spin in the centrifuge is required. Another standard curve should be prepared and centrifuged with the extra samples to correct for variations between spins.)

For all steroids except B:

1. Setting up the samples.

Vortex each tube before aliquoting. Using an Eppendorf P200, pipet 200 μ l of each sample into duplicate assay tubes. With another Eppendorf P200, pipet 100 μ l of the sample into a scintillation vial to count the recoveries. Add 2.5 mls Ultima-Gold scintillation fluid to these vials, cap, vortex, and count on the scintillation counter. (Remember to also count the 2000 cpm vial from Day 1.)

2. Setting up the standard curve.

The standard curve is used to determine the concentration of steroid in the unknown samples (See Appendix C for an explanation of the calculations.) The first three pairs of tubes in the standard curve are the B1-3. B1 measures the total cpm in 100 μ l of the labeled steroid solution. B2 measures non-specific binding (background), and B3 measures the maximum binding of the labeled steroid with the antibody. The remaining 9 pairs of tubes (S1-9) generate the curve.

Add 200 μ l buffer to the B1 and B2 tubes and 100 μ l buffer to the B3 and S2-S9 tubes using the Eppendorf repeater pipette with a 5 ml tip and set on 1. (At this point do not add buffer to the S1 tubes.) Add 100 μ l of cold steroid to each of the S1 and S2 tubes using a Hamilton Syringe. This will be 1000 pg for P, 2000 pg for B, and 500 pg for T, E2, and DHT. Vortex one of the S2 tubes, remove 100 μ l, and add this to an S3 tube. By continuing this serial dilution the concentration will be halved each time. Discard the last 100 μ l that is removed from the S9 tube. Repeat the serial dilution for the second half of the pairs. Go back and add 100 μ l of buffer to all B and S tubes to bring them up to the same volume as the samples.

3. Setting up the radioimmunoassay.

Gently whirl mix (do not shake) the labeled steroid and antibody solutions prior to use. Using the Eppendorf repeater pipette, add 100 μ l of labeled steroid to all of the tubes (standard curve plus samples). Add 100 μ l antiserum to all tubes starting with the B3 tubes (do not add antiserum to the B1 or B2 tubes). Cover with parafilm, whirl mix, and refrigerate overnight. Curves may be left in the refrigerator longer before the charcoal step is done, but overnight is the minimum time required.

For B:

The procedure for B is the same as that described above except that different amounts are used.

1. Setting up the samples.

Aliquot 100 μ l of unknown sample into replicate test tubes, and place 200 μ l in the scintillation vials for the measurement of the recoveries.

2. Setting up the standard curve.

The procedure is identical as above, but do not go back and add 100 μ l of buffer to the B and S tubes following the serial dilution. This is because the unknown aliquots have a volume of only 100 μ l, so the volumes in the curve and in the samples are already identical.

3. Setting up the radioimmunoassay.

Follow the same procedure as described above for the other steroids.

DAY 5

1. Separation of bound and free counts.

Dextran-coated charcoal is added to all of the tubes except the B1's to adsorb any unbound steroid. The tubes are then centrifuged to force the charcoal-dextran to the bottom, and the supernatant, which contains all steroids (hot and cold) bound to antibody, is decanted. The total cpm's in the supernatant are counted. High radioactivity in the supernatant indicates a low concentration of endogenous steroid, while low counts of tritiated steroid indicate high levels of endogenous steroid. The exact concentrations of steroids in the unknown samples are calculated by comparing them to the standard curve (see Appendix C).

- Run the Beckman TJ-6 centrifuge through one complete cycle to cool it to 4°C.

- Add 0.5 mls distilled water to the B1 tubes using an Eppendorf repeater pipette set on 2 with a 12.5 ml tip.

- Place some dextran-coated charcoal in a beaker, add a magnetic stir bar, and place on a stirring plate. The charcoal mix should be stirred continuously while adding 0.5 mls of it to all of the remaining tubes. This step should be done as quickly as possible to avoid heating of the samples and/or the charcoal mix and to ensure that all samples will be subjected to the charcoal mix for the approximately the same amount of time. It may be helpful to cool the beaker and the repeater pipette tips prior to use or to place the charcoal/dextran beaker in an ice bath while aliquoting.

- Whirl mix the racks of tubes and let stand for 12 minutes. Start the timer as soon as the charcoal mix is added to the last sample. This is a critical step, so set a timer and do not get distracted. Load the centrifuge while waiting, being sure to balance the tubes both within and between buckets.

- After the 12 minutes is up centrifuge at 2000 rpm for 10 minutes.

- Decant the supernatant into scintillation vials, add 4.5 mls Ultima-Gold scintillation fluid, cap, vortex and count on the scintillation counter. The centrifuge tubes with the charcoal pellets should be discarded into the radioactive waste.

- To calculate the steroid concentrations of the samples see Appendix C.

AFTER COMPLETION OF ASSAY

All glassware that is used during the assay should be cleaned in a solution of Radiacwash detergent. Prepare a plastic tub with distilled water and the appropriate dilution of the detergent on Day 1. Glassware can be added to the tub as it is used after it is first rinsed with distilled water in the sink in the hood room. Be sure that all solid particles are removed prior to the soaking, and that there are no air bubbles in the submerged glassware. After all glassware has been soaked for at least a day, rinse three times with the tap-distilled water, and then rinse once with double-distilled water. Place the glassware in the drying oven set at 40°C. Higher temperatures can cause adsorption sites to be formed on the glassware. If this happens the glassware should be rewashed.

Radioactive waste, including scintillation vials, should be disposed of properly. Ask either the radioactivity safety personnel or others in the lab to explain the correct procedures to you.

APPENDIX A - YOLK SAMPLING FROM VIABLE EGGS

The following procedure can be used to obtain small yolk samples from viable eggs.

- 1) Use a freshly-laid, unincubated egg. An unincubated egg is used to avoid the development of the embryo, which could potentially synthesize its own hormones.
- 2) Place the egg over a fiberoptic light source. This allows illumination of the egg from underneath so the yolk is visible and avoids overheating of the egg. (It helps to secure a spongy material with a hole in it over the light. Make the hole large enough to just hold the egg in place.)
- 3) Put on some sterile latex gloves.
- 4) Clean the eggshell at the small pole with 70% ethanol and a cotton swab or Q-tip. This will reduce the risk of bacterial infection.
- 5) Remove a sterile Butterfly 27 x 3/8, 8" Tubing Infusion Set (Owens and Minor, 9727 Bauer Dr. East, Indianapolis, IN 46280-1904. Item # 0208-4995-01-01 (\$98.67/120 needles = 1 case, other sizes are available depending on the size of the egg you are sampling)) from its package and attach it to a small syringe. A 1 ml syringe or smaller will work best.
- 6) Use a ruler and a permanent marker to measure off a predetermined volume on the tubing. For this size tubing, 13 mm is equal to 10 μ l.
- 7) Penetrate the eggshell with the tip of the needle in the middle of the small pole of the egg. This will allow you to avoid puncturing the air sac, which is vital to the health of the developing embryo.
- 8) Aim the tip of the needle to the middle of the yolk and penetrate the yolk membranes using a slight push. Too much of a push will force the needle completely through the yolk, while a weak push will not penetrate the yolk membranes. Therefore some practice is necessary. You can test to see if the needle is in the yolk by slightly moving the needle back and forth and looking for movement of the entire yolk.
- 9) Apply suction to the syringe and gently draw the yolk to the mark on the tubing. Too much suction can destroy the yolk membranes and draw albumin, so do not rush.

- 10) Gently withdraw the needle from the egg and place withdrawn yolk into an Eppendorf 1.5 ml tube.
- 11) Add 500 μ l distilled water, vortex vigorously until homogenous and freeze. The addition of two or three small glass beads prior to mixing will facilitate suspension.
- 12) Cut two small patches of OpSite transparent wound dressing (Briggs Corporation, 7887 University Blvd., Des Moines, IA 50306. Item # = 16-4575 (\$1.05/2" x 3" strip)). Remove the paper backing and patch the hole in the shell crosswise. This flexible dressing will allow for the proper exchange of gases while reducing the probability of infection.
- 13) Return the egg to the nest and allow it to develop.

Protocol for Hormone Injections into Egg Yolks

One can use the same procedure as above. The injected volume should be very low. For the canary egg yolk, which has a mass of about 500 mg, the hormone is injected in 5 μ l of sterile sesame oil.

APPENDIX B - SHORT COLUMN CHROMATOGRAPHY

Short chromatography columns may be used if you are not assaying for both B and E2. The differences from the normal protocol are as follows:

- To make the water trap add celite/water mix to the 3.5 ml mark on the column and pack to about 1 mark below the 4.5 ml mark.
- To make the glycol phase add celite/glycol mix to the 3.0 ml mark on the column and pack to the 4.0 ml mark.
- Wet the columns once with 4 ml isooctane. The columns can be allowed to sit overnight at this stage as long as the hoses are attached to the columns and the nitrogen gas is off.
- Add the samples to columns with 0.5 mls of 10% ethyl acetate in isooctane. There is no rinsing step.
- When the samples have been added to the columns and blown down into the celite with nitrogen gas:

Add 2 mls isooctane, blow down, and discard the eluate. (This is the P fraction, but P is better separated and collected on long columns.)

Add 1.5 mls 10% ethyl acetate in isooctane. This is the DHT fraction.

Add 2.0 mls 20% ethyl acetate in isooctane. This is the T fraction.

Add 2.5 mls 50% ethyl acetate in isooctane. This is the B fraction.

- If E2 will be collected instead of B, Add 2.5 mls 40% ethyl acetate in isooctane after the T fraction and skip the B fraction.
- Continue with the normal protocol.

APPENDIX C - CALCULATIONS

Both Mac and DOS-based spreadsheets are available in the lab for the calculation of steroid concentrations in samples. These are self explanatory and are based on the calculations presented below. It is beneficial to calculate sample concentrations by hand and on the computer when learning the assay so that you can understand the logic behind the calculations. A plot of a standard curve (% bound versus log standard concentration) is first created. The amount of steroid in each of the unknown samples is estimated using this curve and the known % bound values for each sample. The concentration of the original yolk sample is then calculated taking into account the recovery values and the dilutions that occurred during the assay.

To draw the curve and calculate the amount of steroid in each unknown sample:

Average the cpm values from the B1-3 and S1-9 pairs. Calculate the % bound of each S sample with the following formula:

$$\% \text{ bound} = \frac{S_x - B2}{B3 - B2}$$

Make a plot of % bound versus steroid concentration (in pg) in each of the S samples. Average the cpm values from each of the unknown samples and calculate the % bound using the formula above. Plug this value into the curve to estimate the pg of steroid in each sample.

To calculate the original yolk steroid concentrations of P, DHT, T, and E2:

Yolk Concentration (pg/mg) = Value from curve (pg) x 2.75 / % recovery / mg yolk used

Yolk Concentration (pg/ml) = Value from curve (pg) x 2.75 / % recovery / μ l yolk used x 1000

To calculate the percent recovery, divide the total cpm (taken from the original scintillation vial with 20 μ l of hot steroid) by 5.5. Divide the cpm values from all recoveries by this value.

To calculate the original yolk steroid concentrations of B:

Yolk Concentration (pg/mg) = Value from curve (pg) x 10 / % recovery / mg yolk used

Yolk Concentration (pg/ml) = Value from curve (pg) x 10 / % recovery / μ l yolk used x 1000

To calculate the percent recovery, divide the total cpm (taken from the original scintillation vial with 20 μ l of hot steroid) by 5.5. Divide the cpm values from all recoveries by this value.

APPENDIX D - REAGENTS

Steroid Assay Buffer - PBSG (0.1 M, pH 7.0)

NaH ₂ PO ₄ : H ₂ O	16.14 g
Na ₂ HPO ₄ : 7H ₂ O	49.05 g
NaCl	27.00 g
Gelatin	3.00 g
NaN ₃	3.00 g
Distilled Water	3 L

Mix in a 4 L jug and place on a heated, magnetic stirring plate. Heat over low heat overnight while stirring until gelatin is dissolved. A temperature of 50°C is adequate. Refrigerate.

Dextran-Coated Charcoal (1x)

Charcoal	2.50 g
Dextran T-70	0.25 g
PBSG	1 L

Place charcoal and dextran in bottle. Add PBSG and stir on a magnetic plate and refrigerate.

APPENDIX E - PREPARATION OF ANTIBODY SOLUTIONS

Testosterone/Dihydrotestosterone: Antibody is purchased from Wien Laboratories, P.O. Box 227, Succasunna, NJ 07876. (201-584-7019). The catalog number is T-3003 and it costs \$35.00 per vial. Each vial makes 100 ml. The vials should be stored frozen at -20°C.

To prepare the antibody solution first thaw the vial. Measure 100 ml PBSG. Pipet a small amount of the buffer into the vial of antiserum and swirl gently. Pipet this into a bottle. Continue this several more times until the vial is completely emptied and rinsed. Add the remainder of the PBSG to the bottle and swirl to mix. Never shake antisera vigorously. Maximum binding is usually around 35%.

Progesterone: Antibody is also purchased from Wien Laboratories. The catalog number is P-1604 and it costs \$30/vial. Each vial makes 50 ml. Store frozen.

Measure 50 ml PBSG and follow the procedure for the T/DHT antibody. Maximum binding is 40-45%.

Estradiol: Antibody is purchased from Arnel, 119 Washington Place, Suite B, New York, NY 10014. (212-620-4622). The catalog number is 1702 and it costs \$195/vial. Each vial makes 1000 ml. Store frozen.

Thaw the vial and add 1 ml distilled water to the vial and swirl to mix. Using a disposable pipet tip, pipet 100 μ l of this solution into a volumetric flask. Bring up to 100 ml with PBSG. Swirl gently to mix. Maximum binding is 40-50%. If it is higher dilute accordingly.

Corticosterone: Antibody is purchased from Endocrine Sciences, 4301 Lost Hills Road, Calabasas Hills, CA 91301. (800-449-9111). The catalog number is B21-42 and it costs \$70/vial for 4 or more vials. You should check the catalog number on the vial upon receipt because they have more than one corticosterone antiserum and they have been known to send the wrong product. Each vial makes 170 ml. Store frozen.

Thaw the vial and add 1.7 ml distilled water. Shake gently by hand to mix. You may remove 1 ml of this solution and add to 100 ml PBSG and freeze the remaining vial contents to be used later. When using the remaining 700 μ l, thaw it and mix with 70 ml PBSG, rinsing with buffer as for the T/DHT antibody. You may also mix the entire 1.7 ml with 170 ml PBSG if you will be doing a lot of assays within a few months. Maximum binding is 45-50%.

APPENDIX F - PREPARATION OF STANDARD STEROID SOLUTIONS

Stock solutions of steroids are made and kept for further dilution in the assay. These steroids are all purchased from Sigma. They are weighed out in a balance away from the assay area and which will not be used to weigh anything that comes into contact with the assay. Weigh 100 mg of steroid onto a small piece of aluminum foil. Carefully fold this up and drop it into a 100 ml volumetric flask. Add 100 ml ethanol, seal with the stopper and parafilm, and swirl to mix. This is very concentrated in terms of the assay and must be handled carefully or contamination can occur.

For DHT, T, and E2: We want a concentration of 500 pg/100 μ l, which is the same as 125 ng/25 ml. In an area away from the assay bench, take 100 μ l of the stock solution (= 100 μ g) and dilute it into 100 mls of distilled water. Discard the pipet tip and all other disposables into a plastic bag for disposal. Mix this solution well. It is now at 1 μ g /ml and contains a total of 1000 ng. Put 125 μ l of this solution into a 25 ml volumetric flask and fill with PBSG. You now have a 500 pg/100 μ l solution. Discard the intermediate solution into a sink outside of the lab, rinse the container several times, and flush the drain with water. The container should be soaked in Radiacwash detergent.

For B: We want a dilution of 2000 pg/100 μ l, which is the same as 500 ng/25 ml. Using the same methods as above, put 100 μ l of the stock solution into 100 ml distilled water and mix

thoroughly. This solution is at 1 µg/ml and contains a total of 1000 ng. Take 500 µl of this solution and mix with 25 ml PBSG in a volumetric flask. You now have a 2000 pg/100 µl solution. Clean up as described above.

For P: You need 250 µl of the intermediate solution in 25 mls of PBSG to obtain a 1000 pg/100 µl solution. Clean up as described above.

APPENDIX G - PREPARATION OF TRITIATED STEROID SOLUTIONS

Tritiated steroids are ordered in 250 µCi vials from New England Nuclear Life Sciences, 549 Albany Street, Boston, MA 02118. (800-551-2121). Prices and catalog numbers are as follows:

Testosterone [1,2,6,7,16,17-3H(N)]	NET-553	\$493
Estradiol [2,4,6,7,16,17-3H(N)]	NET-517	\$377
Corticosterone [1,2,6,7-3H(N)]	NET-399	\$288
Progesterone [1,2,6,7,16,17-3H(N)]	NET-1112	\$334
Dihydrotestosterone [1,2,4,5,6,7-3H(N)]	NET-453	\$744

Using approved safety measure for handling radioactive substances, pipet some pure ethanol into the vial, withdraw the contents and place them into a 10 ml volumetric flask. Continue to rinse and pipet the solution from the vial to the flask several more times. Bring the solution up to 10 mls with ethanol. You now have 250 µCi in 10 mls. To make solutions for the assay you want 10,000 cpm/100 µl. This works out to be about 500 µl of this stock solution in 200 mls PBSG. Remember to rinse the small beaker you pour the stock solution into for measuring as well as the pipet tip itself. Measure the cpm of the working solution on the scintillation counter and adjust accordingly. It is better to add less buffer at the start and then further dilute than to get it too weak and have to add label. All glassware should be soaked in Radiacwash detergent.